

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Andrew VAILLANT et al.
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For: ANTIVIRAL OLIGONUCLEOTIDES
Art Unit: 1648
Examiner: Sharon L. Hurt
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DECLARATION UNDER 37 C.F.R. SEC. 1.132

I, Jean-Marc Juteau, do hereby declare and state as follows:

1. I received the degrees of Bachelor (B.Sc.) of Biology from Montreal University in 1985, Master (M.Sc.) of Microbiology and Immunology from Montreal University in 1988, and Doctor of Philosophy (Ph.D.) of Microbiology and Immunology from Laval University in 1991.
2. My academic background and experiences in the field of the present invention are listed on the enclosed *curriculum vitae*.
3. I am a founder since 1999 of REPLICor Inc. and Senior Vice President since 2003.
4. I am an author of several scholarly publications as listed in my enclosed *curriculum vitae*.
5. I am an inventor in the present application; I have read and am thoroughly familiar with the contents of U.S. Patent Application Serial No. 10/661,403 entitled "ANTIVIRAL OLIGONUCLEOTIDES", including the claims.

6. In order to demonstrate the antiviral activity of selected oligonucleotides (ONs) described in this invention, we tested these ONs against Flaviridae, Hepadnaviridae, Orthomyxoviridae, Paramyxoviridae, Herpesviridae and Filoviridae viruses. The ONs tested were REP 2006, a 40mer phosphorothioate oligodeoxynucleotides having the sequence N₄₀, where N = A, C, T or C (the sequence of REP 2006 is completely degenerate); REP 2031 (SEQ ID NO: 22) is a 40mer phosphorothioate oligodeoxynucleotide having the sequence C₄₀; and REP 2055 (SEQ ID NO: 24) is a 40mer phosphorothioate oligodeoxynucleotide having the sequence (AC)₂₀.

a) Activity in Flaviviridae

The antiviral activity of REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) against Flaviviridae were demonstrated using HCV as the representative flavivirus in infectious *in vitro* and *in vivo* systems.

For *in vitro* testing, monolayers of Huh7.5 hepatocytes were infected with the JFH1 strain of HCV. In this system, infection of the monolayers is monitored using a HCV core antigen ELISA. Huh7.5 cells are also infected with HCV pseudoparticles harbouring a luciferase expression cassette. In this system, infection is monitored by assessing luciferase activity in the tissue culture by standard methods.

Activity of REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) were assessed by adding these compounds to the tissue culture system during infection (see figure 1 below). The results of these tests indicated that both REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) were potently active against HCV infection *in vitro*.

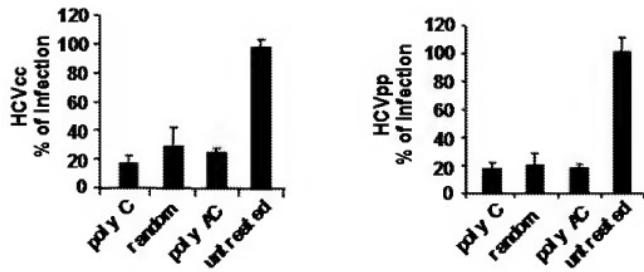


Figure 1. Activity of REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) *in vitro* at 100nM against JFH1 (HCVec, left panel) or pseudoparticle (HCVpp, right panel) strains of HCV infection *in vitro*. Both (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) show potent antiviral activity in this *in vitro* system. Legend: poly C = REP 2031 (SEQ ID NO: 22), random = REP 2006 and poly AC = REP 2055 (SEQ ID NO: 24).

For *in vivo* testing, mice with human/mouse chimeric livers were infected with human HCV positive serum (genotype 1). These chimeric mice support the development of HCV infection in their livers similar to human subjects and HCV infection in this model is known to be treatable with several antiviral compounds including human interferon, and NS3 and NS5 inhibitors currently in clinical trials. In this model, REP 2031 (SEQ ID NO: 22) or REP 2055 (SEQ ID NO: 24) were administered to mice on the day of infection and for an additional 7 days by a once-daily 500 μ l intra-peritoneal bolus injection of either REP 2031 (SEQ ID NO: 22) or REP 2055 (SEQ ID NO: 24) formulated in normal saline at a concentration appropriate to deliver a 10mg/kg dose to subject mice. After the treatment ended, HCV infection was monitored on day 8 by the detection of viremia in blood samples using a RT-PCR based assay specific for HCV virions. The results of these tests (see table 1 below) indicated that both REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) were highly effective in protecting chimeric mice from HCV infection,

further indicating that formulations of REP 2031 and REP 2055 *in vivo* study were suitable for the treatment of human Flaviviridae infections such as HCV.

Table 1

Protective effect of REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) against HCV infection in human chimeric mice.

Treatment	HCV blood titer
Normal saline (placebo)	3/3 mice > 1 log/ml of viral particles
REP 2031 10mg/kg/day for 7 days	3/3 mice: no detectable viral particles
REP 2055 10mg/kg/day for 7 days	3/3 mice: no detectable viral particles

b) **Activity in Hepadnaviridae**

Activity of REP 2006, REP 2031 (SEQ ID NO: 22) or REP 2055 (SEQ ID NO: 24) in Hepadnaviridae were assessed using the representative virus duck hepatitis B virus (duck HBV). For *in vitro* analysis, primary duck hepatocytes are infected with duck HBV and infection is monitored by immunofluorescence detection of duck HBVsAg. Antiviral activity is assessed by a reduction in the percentage of duck HBVsAg positive hepatocytes in the presence of the drug. REP 2006 or REP 2031(SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) were added during infection in the in vitro system at 10nM. Results (see table 2 below)

Table 2

In vitro anti-duck HBV effect of REP 2006 and REP 2031 (SEQ ID NO: 22).

Treatment	% duck HBVsAg positive hepatocytes
PBS	12.8
10nM REP 2006	0.31
10nM REP 2031	3.12

To assess *in vivo* activity, adolescent ducklings were infected with 5×10^8 duck HBV genomes (day 0). Ducklings received placebo (normal saline) or 10mg/kg/day of REP

2006 (N_{40}), REP 2031 (C_{40} , SEQ ID NO: 22) or REP 2055 ($[AC]_{20}$, (SEQ ID NO: 24) in normal saline by daily intraperitoneal injection for 14 days. Duck HBV infection and replication *in vivo* was monitored by assessment on duck HBVsAg immunoreactive cells in primary cultures taken from liver biopsies on day 4 and 14.

The following table summarizes the ability of selected ONs to inhibit duck HBV infection / replication *in vivo* in adolescent ducklings.

Table 3

Percentage of infected (DHBVsAg positive) hepatocytes in DHBV infected ducklings.

Day	Normal saline	REP 2006	REP 2031	REP 2055
4	2.728	0.006	0.429	0.0008
14	>95	<0.001	>95	<0.0003

These results demonstrate that REP 2006, REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) display *in vitro* and *in vivo* anti-Hepadnaviridae activity and are suitable for use in methods of treatment of Hepadnaviridae infections such as HBV in human patients.

c) Activity in Orthomyxoviridae

Influenza A species were used as representative of the Orthomyxoviridae family in assessing activity of REP 2006, REP 2031 (SEQ ID NO: 22) in infectious *in vitro* and *in vivo* systems.

MDCK cells were exposed to viruses and after infection, the progression of influenza infection was monitored by the addition of a 0.5% suspension of chicken erythrocytes (CEs). When the erythrocytes added to the tissue culture and antiviral control wells

were totally settled, the pattern of hemagglutination in every well was observed and recorded. Tight buttons of CEs were seen in wells with no antiviral activity, while the CEs in wells with virus-induced CPE exhibited a diffuse pattern. The mean concentration of compound in the last wells of replicate rows in which growth of virus was inhibited in 50% of the wells (the IC₅₀) was then determined. Results (see table 4 below) indicate that REP 2006 and REP 2031 (SEQ ID NO: 22) exhibited potent antiviral activity against influenza A infection *in vitro*.

Table 4
Anti-influenza activity of REP 2006 and REP 2031 *in vitro*

Compound	IC ₅₀ (uM)
REP 2006	0.008
REP 2031	0.002

For *in vivo* evaluation of REP 2006 or REP 2031 (SEQ ID NO: 22) activity against influenza A/H5N1 infection *in vivo*, mice were exposed daily (from day -1 to day 16) to REP 2006 or REP 2031 (SEQ ID NO: 22) via simultaneous intranasal (5mg/kg/narc formulated in water) and intraperitoneal (10mg/kg formulated in normal saline) administration. Mice were intranasally infected on day 0 with 1000 LD50 of influenza A/Vietnam/1203/04. Mice were followed for morbidity and mortality until day 21 at which protected mice were considered to have recovered from the infection. Mice were determined to be ill if classical signs of illness were observed. These included ruffled fur, hunched posture, decreased mobility or signs of neurologic disease such as paralysis, tremors or seizures. Moribund mice were euthanized and counted as dead on the subsequent day.

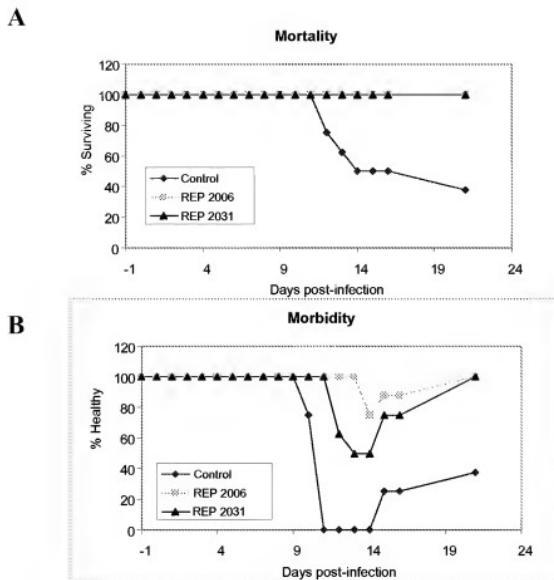


Figure 2. Protective effect of REP 2006 and REP 2031 (SEQ ID NO: 22) against Influenza A infection in mice. Effects of REP 2006 and REP 2031 (SEQ ID NO: 22) on mortality (A) and morbidity (B) in a lethal A/Vietnam/1203/04 (H5N1) infection demonstrate that antiviral activity of these compounds against influenza infection *in vivo*.

These data clearly show that REP 2006 or REP 2031 (SEQ ID NO: 22) are potently effective both *in vitro* and *in vivo* in treating Orthomyxoviridae infections such as influenza A infections and thus are well suited for the treatment of such infections in human patients.

d) Activity in Paramyxoviridae.

REP 2006 and REP 2031 (SEQ ID NO: 22) antiviral activity against Paramyxoviridae were assessed using the representative paramyxovirus respiratory syncytial virus (RSV).

In vitro evaluations of both REP 2006 and REP 2031 (SEQ ID NO: 22) *in vitro* were carried out using the same *in vitro* methodology for influenza virus except that RSV (strain A2) was used to infect cells. Table 5 (below) demonstrates that REP 2006 and REP 2031 (SEQ ID NO: 22) are potently active against RSV infection *in vitro*.

Table 5

Antiviral efficacy of REP 2006 and REP 2031 against RSV infection *in vitro*.

Compound	IC ₅₀ (uM)
REP 2006	0.0025
REP 2031	0.0017

These data clearly demonstrate the efficacy of both REP 2006 and REP 2031 (SEQ ID NO: 22) against RSV infections *in vitro*.

e) Activity in Herpesviridae

Representative viruses used to assess the activity of REP 2006, REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) in Herpesviridae are HSV-1, HSV-2 and CMV. *In vitro* assessment of REP 2006, REP 2031 (SEQ ID NO: 22) and REP 2055 (SEQ ID NO: 24) *in vitro* were performed by adding compounds during viral infection after which cells (VERO cells) were washed and replaced with overlay media. IC₅₀ values were calculated as the concentration of compound which reduced the number of plaques by 50%

compared to the untreated control. Test compounds were added during infection and throughout the rest of the assay. *In vitro* efficacy results are summarized in table 6 below.

Table 6

Efficacy of REP 2006, REP 2031 and REP 2055 in Herpesviridae infection in vitro.

Compound	HSV-1 IC ₅₀ (uM)	CMV IC ₅₀ (uM)
REP 2006	0.136	0.02
REP 2031	0.097	Not determined
REP 2055	0.123	Not determined

Compounds were further assessed for *in vivo* activity topically against HSV-2 and by parenteral administration against CMV liver infection (see table 7 and figure 3 below). For anti-CMV assessment *in vivo*, compounds were administered on days -3, -2, -1, 0, 1 and 2 with infection with on day 0 with CMV (strain Smith) by intraperitoneal injection (10mg/kg of REP 2006 and REP 2031, SEQ ID NO: 22) formulated in normal saline. Ganciclovir (GCV) was used as a positive control. Titers were determined by plaque assay from spleens harvested 3 days after infection. For HSV-2 assessment *in vivo*, HSV-2: female Swiss Webster mice were administered a 0.1 ml suspension containing 3 mg of medroxyprogesterone acetate by subcutaneous injection 7 and 1 days prior to viral challenge, to increase susceptibility to vaginal HSV-2 infection. Animals were treated vaginally with 15 µl of either the candidate solution (REP 2006 or REP 2031, SEQ ID NO: 22) formulated in PBS or a placebo control using a positive displacement pipetter. Five minutes later, animals were inoculated by instillation of 15 µl of a suspension containing HSV-2, strain 186. Mice were evaluated daily up to day 21 after inoculation, for evidence of symptomatic infection that can include hair loss and erythema around the perineum, chronic urinary incontinence, hind-limb paralysis, and mortality. Animals that

did not develop symptoms were defined as infected if virus was isolated from vaginal swab samples collected on day 2 after inoculation.

Table 7
Protection of animals from HSV-2 transmission by REP 2006 and REP 2031 (SEQ ID NO: 22).

Compound	HSV-2 protection
Placebo (PBS)	0/12 animals protected
REP 2006	8/12 animals protected ($p=0.001$)
REP 2031	12/12 animals protected ($p=0.001$)

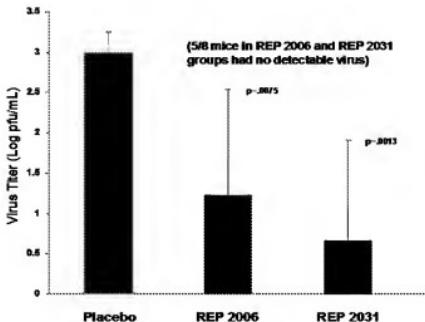


Figure 3. IP administration of either REP 2006 or REP 2031 (SEQ ID NO: 22) significantly inhibits CMV liver replication *in vivo*.

These results demonstrate that REP 2006, REP 2031 and REP 2055 are effective agents in treating Herpesviridae infections and may be useful to treat these infections in human subjects.

f) Activity in Filoviridae

The representative Filoviridae used to test the activity of REP 2006 and REP 2031 (SEQ ID NO: 22) is Ebola (strain Zaire expressing GFP).

For *in vivo* analysis, mice were treated during infection with a lethal dose of Ebola Zaire. REP 2006 or REP 2031 (SEQ ID NO: 22) treatment was daily by intra-peritoneal injection with the compounds formulated in normal saline to give a daily dose of 20mg/kg. Compound efficacy was monitored by survival of mice from the lethal infection. Several different treatment regimens (prophylactic and therapeutic) were assessed, see figure 4 below.

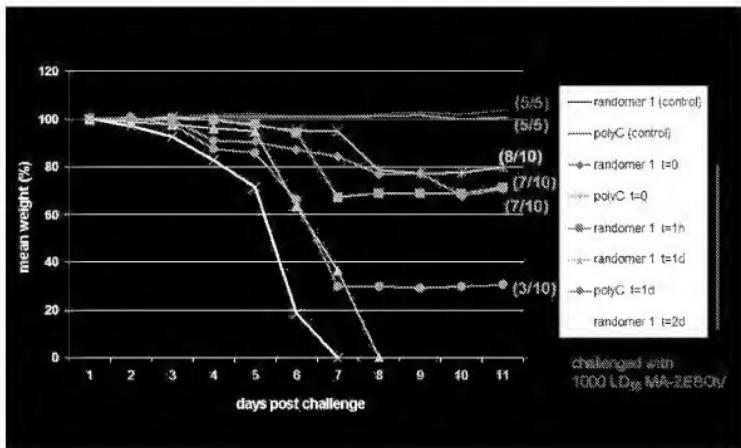


Figure 4. Protection of mice from lethal Ebola infection by REP 2006 (randomer 1) or REP 2031 (SEQ ID NO: 22; poly C). Time indicated how long after infection treatment was started. Note that REP 2006 given 2 days after infection showed no difference from control untreated animals, however all other treatment regimens show moderate to highly potent protective activity.

These data demonstrate that REP 2006 and REP 2031 (SEQ ID NO: 22) are effective agents against Filoviridae infection such as Ebola *in vivo* and may be useful for the treatment of such infections in human patients.

7. The results presented above and produced according to the teaching of the present invention clearly proves that the present invention has clinical relevance and in addition, that the *in vitro* results disclosed in the present application do not diverge from *in vivo* responses.
8. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both (18 U.S.C. Sec. 1001), and may jeopardize the validity of the application of any patent issuing thereon.

Signed



Jean-Marc Juteau

Dated: June 19, 2007